NUCLEOTIDE SEQUENCES CODING FOR POLYPEPTIDES ENDOWED WITH A
LARVICIDAL ACTIVITY TOWARDS LEPIDOPTERA

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5   The subject of the invention is nucleotide sequences coding
for polypeptides endowed with a larvicidal activity towards
Lepidoptera.

           It relates more particularly to agents, in particular
nucleotide sequences, polypeptides or even vectors, or bacterial

10   strains modified by these sequences and expressing polypeptides making
it possible to prepare larvicidal compositions active against
Lepidoptera, preferably against Spodoptera littoralis (hereafter
S.littoralis) or Mamestra brassicae (hereafter designated by
M.brassicae) or capable of transforming the plants to be treated in

15   confe.ring on them this type of activity.

           It is known that most of the isolates of B.thuringiensis
show a toxic activity with regard to larvae of more than a hundred
species of Lepidoptera.

           This activity results from the capacity of the strains of

20   B.thuringiensis to synthesize, at the moment of sporulation,
crystalline inclusions of protein nature, or δ-endotoxins, under the
control of one or several types of gene.

           It has been shown that the activity of these polypeptides
is contained in the NH_2-terminal half or N-terminus of the protein.

25   The studies carried out have shown the high specificity
of the δ-endotoxins towards larvae of a given species.

           On account of this high specificity, many species of
Lepidoptera, in particular of the family of the Noctuidae, react only
weakly to commercial preparations of available B.thuringiensis.

30   It is so in particular for the species S.littoralis, a poly-
phagous insect which constitutes the principal parasite of cotton
and other industrially important crops. Among these crops, mention
should be made of maize, the castor oil plant, tobacco, the groundnut,
fodder plants, such as clover or alfalfa, or also market garden produce

35   such as the cabbage or the tomato.

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Hence, one can imagine the interest of disposing of agents targeting specifically and effectively the family of the Noctuidae and in particular S.littoralis or M.brassicae.

The genes for δ-endotoxins hitherto identified do not code
5   for a polypeptide preferentially active with regard to S.littoralis.

The search by the inventors for a sequence of nucleotides coding for a polypeptide preferably active against the Noctuidae, more especially against S.littoralis, has led them to study the natural isolates of two strains of B.thuringiensis, the larvicidal activity
10 of which on S.littoralis appears to be higher than that of the industrial preparations made starting from other strains of B.thuringiensis.

The species in question are aizawai 7-29 and entomocidus 6-01.

15 The study of these isolates has made it possible to demonstrate the existence of several genes for δ-endotoxins of different structures and different specificities, of which two genes preferentially active against P.brassicae but not very active against the Noctuida of cotton and a gene inactive against P.brassicae and
20 S.littoralis.

By studying the total DNA of these isolates and by carrying out appropriate hybridizations, followed by the cloning of the fragments identified by hybridization, the inventors have observed that it is possible to isolate nucleotide sequences implicated in
25 genes for δ-endotoxins coding for polypeptides active, preferably, against S.littoralis.

Thus, the aim of the invention is to provide nucleotide sequences capable of coding for at least the NH_2-terminal part of a δ-endotoxin toxic against the Noctuidae and preferably against
30 S.littoralis or M.brassicae.

It also has the aim of providing a polypeptide toxic with regard to the Noctuidae.

Furthermore, the invention relates to a procedure for obtaining such a sequence and a polypeptide showing the desired
35 activity as well as the intermediate agents such as vectors and

bacterial strains which can be utilized for obtaining the polypeptide.

In addition, the invention relates to the uses of these
sequences and polypeptides for the development of larvicidal
compositions with regard to the Noctuidae, in particular S.littoralis
and for the transformation of the plants likely to be infected by
these larvae.

The invention relates to a sequence of nucleotides coding
for at least a part of the N-terminal region of a polypeptide toxic
specifically against the larvae of Lepidoptera of the Noctuidae family,
and preferably against S.littoralis, characterized by its capacity
of hybridization with a gene capable of expressing a polypeptide
toxic towards larvae of S.littoralis.

According to another aspect of the invention, the nucleotide
sequence is characterized in that it is carried by a sequence of
nucleotides of about 3 kb such as obtained by in vitro genetic
recombination of sequences of nucleotides of B.thuringiensis capable
of hybridizing with probes 1, 2 and 3 of pHTA2 shown in figure 2.
The fragment of 3 kb corresponds more particularly to the restriction
fragment HindIII-PstI.

The sequences of nucleotides of the invention are, in
addition, characterized in that they contain sites in the following
order : HindIII - HincII - BglII - KpnI - HindIII - PstI.

In a preferred manner, these sequences of nucleotides are
obtained by in vitro genetic recombination of DNA sequences derived
from at least one strain of B.thuringiensis. In a variant of the
embodiment of the invention, two different strains of B.thuringiensis
are utilized.

Strains of B.thuringiensis particularly suited for obtaining
these sequences of nucleotides are the strains corresponding to aizawai
7-29 and entomocidus 6-01, deposited on 21 April 1987 under the No.
I-661 and No. I-660, respectively, with the National Collection of
Cultures of Microorganisms (N.C.C.M.) in Paris.

In an advantageous manner, the sequences of nucleotides
of the invention code for a polypeptide capable of forming an
immunological complex with antibodies directed against polypeptides
showing the larvicidal activity with regard to S.littoralis.

A sequence of nucleotides according to the invention is characterized in that it has the capacity to hybridize with a probe formed from the sequence (I) showing the following chain arrangement:

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     52
GTC TAC TTG ACA GGG GTA GCA ACA TAA TCG GTC AAT TTT AAA
                              112
TAT GGG GCA TAT ATT GAT ATT TTA TAA AAT TTG TTA CGT TTT
                                                      172
TTG TAT TTT TTC ATA AGA TGT GTC ATA TCT ATT AAA TCG TGG

TAA TGA AAA ACA GTA TCA AAC TAT CAG AAC TTT GGT AGT TTA
                    232
ATA AAA AAA CGG AGG TAT TTT ATG GAG GAA AAT AAT CAA AAT
                                              292
CAA TGC ATA CCT TAC AAT TGT TTA AGT AAT CCT GAA GAA GTA

CTT TTG GAT GGA GAA CGG ATA TCA ACT GGT AAT TCA TCA ATT
            352
GAT ATT TCT CTG TCA CTT GTT CAG TTT CTG GTA TCT AAC TTT
                                    412
GTA CCA GGG GGA GGA TTT TTA GTT GGA TTA ATA GAT TTT GTA

TGG GGA ATA GTT GGC CCT TCT CAA TGG GAT GCA TTT CTA GTA
    472
CAA ATT GAA CAA TTA ATT AAT GAA AGA ATA GCT GAA TTT GCT
                        532
AGG AAT GCT GCT ATT GCT AAT TTA GAA GGA TTA GGA AAC AAT
                                                  592
TTC AAT ATA TAT GTG GAA GCA TTT AAA GAA TGG GAA GAA GAT

CCT AAT AAT CCA GAA ACC AGG ACC AGA GTA ATT GAT CGC TTT
                        652
CGT ATA CTT GAT GGG CTA CTT GAA AGG GAC ATT CCT TCG TTT
                                              712
CGA ATT TCT GGA TTT GAA GTA CCC CTT TTA TCC GTT TAT GCT

CAA GCG GCC AAT CTG CAT CTA GCT ATA TTA AGA GAT TCT GTA
            772
ATT TTT GGA GAA AGA TGG GGA TTG ACA ACG ATA AAT GTC AAT
                                    832
GAA AAC TAT AAT AGA CTA ATT AGG CAT ATT GAT GAA TAT GCT

GAT CAC TGT GCA AAT ACG TAT AAT CGG GGA TTA AAT AAT TTA
    892
CCG AAA TCT ACG TAT CAA GAT TGG ATA ACA TAT AAT CGA TTA
                        952
CGG AGA GAC TTA ACA TTG ACT GTA TTA GAT ATC GCC GCT TTC

TTT CCA AAC TAT GAC
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Sequences of nucleotides coding for at least a part of the
N-terminal region of a polypeptide toxic specifically towards larvae
of Lepidoptera of the Noctuidae family, and preferably towards
S.littoralis, are characterized in that they contain the chain
5   arrangement (I) defined above.

In an advantageous manner, the sequence of nucleotides
characterized by the chain arrangement defined above codes for a part
of a polypeptide having a higher larvicidal activity towards
S.littoralis than that of the polypeptides encoded by natural isolates
10   presently known for their effects against S.littoralis.

The study of this sequence of nucleotides shows that it
is characterized by an initiation codon ATG situated at position 241
starting from which an open reading frame of 750 nucleotides has been
identified.

15   This sequence is also characterized by a GGAGG attachment
site for ribosomes at positions 230 to 234.

According to another feature, the sequence of nucleotides
of the invention is characterized in that it contains, upstream from
the ATG codon, a sequence going from the nucleotide at position 137
20   to the nucleotide at position 177, strongly homologous with the region
found by Wong et al. (1983) and described in (16) upstream from the
gene for the crystal of the strain kurstaki HD1 Dipel (BTK) and for
which the authors have shown that it contains three promoters BtI,
BtII and Ec which are functional in B.thuringiensis and E.coli,
25   respectively. The homology of these sequences is about 70%.

The invention also relates to a sequence of nucleotides
coding for the following sequence (II) of amino acids :

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                                    MET GLU GLU ASN ASN GLN ASN
GLN CYS ILE PRO TYR ASN CYS LEU SER ASN PRO GLU GLU VAL
LEU LEU ASP GLY GLU ARG ILE SER THR GLY ASN SER SER ILE
ASP ILE SER LEU SER LEU VAL GLN PHE LEU VAL SER ASN PHE
VAL PRO GLY GLY PHE LEU VAL GLY LEU ILE ASP PHE VAL TRP
GLY ILE VAL GLY PRO SER GLN TRP ASP ALA PHE LEU VAL GLN
ILE GLU GLN LEU ILE ASN GLU ARG ILE ALA GLU PHE ALA ARG
ASN ALA ALA ILE ALA ASN LEU GLU GLY LEU GLY ASN ASN PHE
ASN ILE TYR VAL GLU ALA PHE LYS GLU TRP GLU GLU ASP PRO
ASN ASN PRO GLU THR ARG THR ARG VAL ILE ASP PRG PHE ARG
ILE LEU ASP GLY LEU LEU GLU ARG ASP ILE PRO SER PHE ARG
ILE SER GLY PHE GLU VAL PRO LEU LEU SER VAL TYR ALA GLN
ALA ALA ASN LEU HIS LEU ALA ILE LEU ARG ASP SER VAL ILE
PHE GLY GLU ARG TRP GLY LEU THR THR ILE ASN VAL ASN GLU
ASN TYR ASN ARG LEU ILE ARG HIS ILE ASP GLU TYR ALA ASP
HIS CYS ALA ASN THR TYR ASN ARG GLY LEU ASN ASN LEU PRO
LYS SER THR TYR GLN ASP TRP ILE THR TYR ASN ARG LEU ARG
ARG ASP LEU THR LEU THR VAL LEU ASP ILE ALA ALA PHE PHE
PRO ASN TYR ASP
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7

A better identification of the sequence of nucleotides
isolated from the above strains, deposited with the NCCM has made
it possible to observe that the nucleotide situated at position 273
is guanine (G), the amino acid resulting from the GTA codon thus being

5   valine.

Now, the reading of the nucleotide corresponding to this
position 273 in the application FR.8708090 of 10 June 1987 had led
to reporting thymine (T) and leucine as amino acid resulting from
the TTA codon.

10   Another sequence of nucleotides of the invention is
characterized by its capacity of hybridization with a probe formed
from the sequence (III) showing the following chain arrangement:

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AAG CTT CAA TAG AAT CTC AAA TCT CGA TGA CTG CTT AGT CTT TTT AAT ACT GTC TAC TTG ACA GGG GTA GGA ACA TAA TCG GTC AAT TTT

AAA TAT GGG GCA TAT ATT GAT ATT TTA TAA AAT TTG TTA CGT TTT TTG TAT TTT TTC ATA ACA TGT GTC ATA TGT ATT AAA TCG TGG TAA

TCA AAA ACA GTA TCA AAC TAT CAG AAC TTT GGT ACT TTT ATA AAA ACG AGG TAT TTT ATG GAG GAA AAT CAA AAT TGC GAA

CCT TAC AAT TGT TTA AGT AAT CCT GAA GAA GTA CTT TTG GAT GGA GAA GGG ATA TCA ACT GGT AAT TCA TCA ATT GAT ATT TCT CTG TCA

CTT GTT CAG TTT CTA GTA TCT AAC TTT GTA CCA GGG GGA TTT TTA GTT GGA TTA ATA GAT TTT GCT AGG AAT ATA GCT ATT GCT ATT TTA GAA

CAA TGG CAT GCA TTT CTA GTA CAA ATT GAA CAA TTA ATT AAA ATA TAT GTG GAA TTT AAA GAA GCA TTT AAA GAA GAA TTT GCT AGG AAT GCT GGA ATA GTT GGC CCT TCT

GGA TTA GGA AAC AAT TTC AAA ATA TAT GTG GAA GCA TTT AAA GAA GGA CCT AAT AAT CCA GCA ACC AGG ACC AGA GTA ATT

GAT CGC TTT CGT ATA CTT GAT GGG CTA CTT GAA AGG GAC ATT CCT TCG TTT CGA ATT TCT GGA ATA CCC CTT TTA TCC GTT TAT

GCT CAA GCC GCC AAT CTG CAT CTA GCT ATA TTA AGA GAT TCT GTA ATT TTT GGA GAA AGA TGG GGA TTG ACA ACG ATA AAT GTC AAT GAA

AAC TAT AAT AGA CTA ATT AGG CAT ATT GAT GAA TAT GCT GAT GAT CAC TGT GCA AAT ACG TAT AAT ACG TAT AAT CGG GGA TTA AAT AAT TTA CCG AAA TCT

ACC TAT CAA GAT T G ATA ACA TAT AAT CGA TTA CGG AGA GAC TTA ACA TTG ACT GTA TTA GAT ATC GCC GCT TTC TTT CCA AAC TAT GAC

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991
AAT AAG ACA TAT CCA ATT CAG CCA GTT GGT CAA CTA ACA AGG GAA GTT TAT ACG GAC CCA TTA ATT AAT TTT AAT CCA CAG TTA CAG TCT
1061

GTA CCT CAA TTA CCT ACT TTT AAC GTT ATG GAG GAG AGC AGC GCA ATT AGA AAT CCT CAT TTA TTT GAT ATA TTG AAT AAT CTT ACA ATC TTT
1131

ACG GAT TGG GTT AGT GTT GGA CGC AAT TTT TAT TGG GGA CAT CCA ATA TCT AGC CTT ATA GGT GGT AAC ATA ACA TCT CCT
1201

ATA TAT GGA GAG GCG AAC GAG CAG CCG AAC GCC TTT CCA AGA TCC TTT ACT TTT AAT GAA CCA GTA ATT TCA ATT CCT ACT TTA CGA
1271

TTA TTA CAG CAA CCT TGC CAG CGC CAC CAT TTT AAT TTA CGT GGT GGT GAA GGA TTT TCT ACA CCT AGC AAT AGC TTT ACG TAT
1341

CGA GGA AGA GGT ACG GTT GAT TCT TTA ACT TCT GGA ACA TCT TTA CCG CCT CGC GAA GAT AAT AGT GTG CCA CTT CGT AGT CAT CTT TTA TGT
1411

CAT GCA ACT TTT GTT CAA AGA TCT GGA ACA CCT TTT TTA ACT GTA ACT CCT TTT TCT TGG ACG CAT CGT GGT GCA ACT CTT ACA AAT
1481

ACA ATT CAT CAA GAG AGA ATT AAT CAA ATA CCT AGG ACT TTA GTG AAA GAA TTT AGA GGC ACC TCT GTC ATT ACA GGA CCA GGA TTT
1551

ACA CAA CAG CAT ATC CTT CGA AGA AAT ACC TTT CGA GAT TTT GGT GAT TCT CTA CAA ATT TCA CCA ATT ACC CAA AGA TAC CGT
1621

TTA AGA TTT CGT TAC GCT TCC AGT AGG GAT GCA CAA GTT ATA GTA TTA ACA GAA GTG GAA AAC CAA GTT ACT ATA
1691
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1661
GAT ATG CCT CTT CAG AAA ACT ATG GAA ATA GGG TGG AAC TTA ACA TCT AGA ACA TTT AGA TAT ACC GAT TTT AGT AAT CCT TTT TCA TTT
1741

GCG GCT AAT CCA GAT ATA ATT GGG ATA AGT GAA CAA CCT CTA TTT GGT GCA GAT TCT ATT AGT AGC GGT GAA CTT TAT ATA GAT AAA ATT
2071

GAA ATT ATT CTA GCA GAT GCA ACA TTT GAA GCA ACA TCT GAT TTA GAA GCA AAA GCG GTG AAT GCC CTG TTT ACT TCT TCC AAT
2161

CAA ATC GGG TTA AAA ACC GAT GTG ACG GAT TTA TCA TAT CAT ATT GAT CAA GTA TCC AAT TTA GTG GAT TGT TTA TCA GAT GAA TTT TGT CTG GAT
2251

GAG AAG TTA GAA TTG TCC GAG AAA GTC AAG CCA CTC AGT GAT GAG CAG AAT TTA CTT CAA GAT CCA AGC TTC AGA GGG ATC
2341

AAT AGA CAA CCA GAC CGT GGC TGG AGA GGA AGT ACA GAT ATT ACC CAA GGA GGA GAT GAC GTA TTC AAA GAG AAT TAC GTC ACA CTA
2431

CCG GGT ACC GTT GAT GAG TGC TAT TAT CCA ACG TAT TTA TAT CCA ACG TAT TTA CAG AAA ATA GAT GAA TCG AAA TTA ACC CGT TAT GAA TTA AGA
2521

GGG TAT ATC GAA GAT AGT CAA GAC TTA GAA ATC TAT TTG ATC GCG TAC ATT GAA AGA GAA ATA GTA AAT GTG CCA GAT GGC ACG GGT TCC
2611

TTA TGG CCG CTT TCA GCC CAA AGT CCC CAA ATC GGA AAG TGT GGA GAA CCG AAT CAA TGC GCG CCA CAC CTT GAA TGG AAT CCT GAT CTA GAT
2701

TGT TCC TGC AG
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11

In a distinctive manner, sequences of nucleotides of the
invention coding for a polypeptide toxic specifically towards larvae
of Lepidoptera of the Noctuidae family, and preferably toward
S.littoralis comprise or are reconstituted by the chain arrangement (III)
previously defined.

The chain arrangement (III), comprised in the sequence of
nucleotides of the invention contains 2711 nucleotides. This fragment
includes in particular the potential promoter of the gene of the
δ-endotoxin active on S.littoralis.

Sequences of nucleotides modified in relation to the chain
arrangements (I) or (III) described above naturally enter into the
framework of the present invention to the extent to which these
modifications do not generate appreciable variations of the toxicity
of the polypeptide coded by the modified sequence towards S.littoralis.

These modifications may, for example, consist of deletions,
substitutions, recombinations.

Thus, the sequences of nucleotides (I) and (III) contain
at their position 611 a variable nucleotide corresponding to adenine
(A) in the sequence (I) and to cytosine (C) in the sequence (III).
These nucleotides enter into the composition of the respective codons
GAA and GCA which code respectively for the amino acids glutamic acid
(GLU) and alanine (ALA) in the respective sequences II and IV.

Similarly, any sequence of nucleotides which can hybridize
with that of the chain arrangements (I) or (III) such as obtained
by reverse enzymatic transformation of the corresponding RNA or even
by chemical synthesis also enter into the framework of the definitions
of the invention.

The sequence of nucleotides of formula (III) starts with
a ATG initiation codon situated at position 241 and which represents
the start of an open reading frame of 2470 nucleotides.

The invention also relates to a sequence of nucleotides
characterized in that it codes for a polypeptide containing the
sequence (IV) of amino acids below:

MET GLU GLU ASN ASN GLN ASN GLN CYS ILE

271
PRO TYR ASN CYS LEU SER ASN PRO GLU GLU VAL LEU LEU ASP GLY GLU VAL ILE SER THR GLY ASN SER SER ILE ASP ILE SER LEU SER

301
LEU VAL GLN PHE LEU VAL SER ASN PHE VAL PRO GLY GLY GLY PHE LEU ILE ASP PHE VAL TRP GLY ILE GLY VAL GLY PRO SER

431
GLN TRP ASP ALA PHE LEU VAL GLN ILE GLU GLN LEU ILE GLU GLN ARG ASN ALA ALA ILE ALA ASN LEU GLU

341
GLY LEU GLY ASN PHE ASN ILE TYR VAL GLU ALA PHE LYS GLU TRP GLU ALA ASP PRO ALA THR ARG VAL ILE

631
ASP ARG PHE ARG ILE LEU ASP GLY LEU LEU GLU ARG ASP ILE PRO SER PHE GLU VAL PRO LEU LEU SER VAL TYR

221
ALA GLN ALA ALA ASN LEU HIS LEU ALA ILE LEU ARG ASP SER VAL ILE PHE GLY LEU LEU THR ILE ASN VAL ASN GLU

611
ASN TYR ASN ARG LEU ILE ARG HIS ILE ASP GLU TYR ALA ASP HIS CYS ALA ASN THR TYR ASN ARG GLY LEU ASN ASN LEU PRO LYS SER

301
THR TYR GLN ASP TRP ILE THR TYR ASN ARG LEU ARG ARG ASP LEU THR VAL LEU ASP ILE ALA ALA PHE PHE PRO ASN TYR ASP

991   ASN ARG ARG TYR PRO ILE GLN PRO VAL VAL GLY GLN LEU THR ARG GLU VAL TYR THR ASP PRO LEU ILE ASN PHE ASN PRO GLN LEU GLN SER

1061   VAL ALA GLN LEU PRO THR PHE ASN VAL MET GLU SER SER ALA ILE ARG ASN PRO HIS LEU ASN ASN LEU THR ILE PHE

1171   THR ASP TRP PHE SER VAL GLY ARG ASN PHE TYR TRP GLY GLY HIS ARG VAL ILE SER SER LEU ILE GLY GLY ASN ILE THR SER PRO

1261   ILE TYR GLY ARG GLU ALA ASN GLN GLU PRO PRO ARG SER PHE THR PHE ASN GLY PRO VAL PHE ARG THR LEU SER ILE PRO THR LEU ARG

1331   LEU LEU GLN GLN GLN PRO CYS GLN ARG HIS HIS PHE ASN LEU ARG GLY GLY LEU GLU GLY VAL GLU PHE SER THR PRO THR ASN SER PHE THR TYR

1441   ARG GLY THR ARG GLY THR VAL ASP SER LEU THR GLU GLY LEU PRO PRO ARG GLU GLY TYR SER HIS ARG LEU CYS

1531   HIS ALA THR PHE VAL GLN ARG SER GLY THR PRO PHE LEU THR THR GLY VAL VAL PHE SER TRP THR HIS ARG SER ALA THR LEU THR ASN

1621   THR ILE ASP PRO GLU ARG ILE ASN GLN ILE PRO LEU VAL CYS GLY PHE ARG VAL TRP GLY THR GLY THR SER VAL ILE THR GLY PRO GLY PHE

1711   THR GLY GLY ASP ILE LEU ARG ARG ASN THR PHE GLY ASP PHE VAL SER LEU GLN VAL ASN SER PRO ILE THR ASN ARG GLN ARG TYR ARG

1801   LEU ARG PHE ARG TYR ALA SER SER ARG ASP ALA ARG VAL ILE VAL LEU THR GLY ALA ALA SER THR GLY VAL GLY VAL GLN VAL SER VAL

1124
ASN MET PRO LEU GLN LYS THR MET GLU ILE GLY GLU ASN LEU THR PHE ARG TYR PHE ARG TYR ASP PHE SER.ASN PRO PHE SER PHE

1181
ARG ALA ASN PRO ASP ILE ILE GLY ILE SER GLU GLN PRO LEU PHE ALA GLY SER ILE SER GLY GLU LEU TYR ILE ASP LYS ILE

2071
GLU ILE ILE LEU ALA ASP ALA THR PHE GLU ALA.GLU SER ASP LEU GLU ARG ALA GLN LYS ALA VAL ASN ALA LEU PHE THR SER SER ASN

2181
GLN ILE GLY LEU LYS THR ASP VAL THR ASP VAL SER ASN ALA LEU VAL SER ASN LEU VAL SER ASN ASP GLU PHE CYS LEU ASP

2251
GLU LYS ARG GLU LEU SER GLU GLU LYS VAL LYS HIS ALA LYS ARG LEU SER ASP.GLU ARG ASN LEU LEU GLU ASP PRO ASN PHE ARG GLY ILE

2341
ASN ARG GLN PRO ASP ARG GLY TRP ARG GLY SER THR ASP ILE THR GLN GLY ASP VAL ASP ASP VAL PHE LYS GLU ASN TYR VAL THR VAL THR LEU

2421
PRO GLY GLY THR VAL ASP GLU CYS TYR PRO THR TYR GLN LYS ILE ASP GLU SER LYS LEU LYS ALA TYR THR ARG TYR GLU LEU ARG

2521
GLY TYR ILE ILE GLU ASP SER GLN ASP LEU GLU ILE GLU ILE TYR LEU ILE ALA LYS HIS GLU ILE VAL ASN VAL PRO GLY THR GLY SER

2811
LEU TRP PRO LEU LEU SER ALA GLN SER PRO ILE GLY LYS CYS GLY GLU PRO ASN ARG CYS ALA PRO HIS LEU GLU TRP ASN PRO ASP LEU ASP

271
CYS SER CYS

The invention also relates to recombinant expression and
cloning vectors comprising more particularly at least one sequence
of nucleotides such as that defined above, in particular at least
a part of the sequence of about 3 kb.

A specific recombinant vector is, for example, a plasmid
containing the HindIII-PstI fragment of the sequence of nucleotides
of the invention, inserted in a vector pUC9. A first preferred vector
is the plasmid pHT71, the construction of which is reported in the
assemblies below, which comprises a HindIII-PstI DNA fragment according
to the invention constituted uniquely of DNA derived from the strain
aizawai 7-29.

Another recombinant vector is constituted by the plasmid
pHT 671, the construction of which is given in figure 4. This plasmid con-
tains a chimeric HindIII-PstI fragment, obtained by fusing a HindIII-
HindII fragment of 1.1 kb derived from the strain entomocidus 6-01
with a HincII-PstI fragment of 1.9 kb derived from the strain aizawai
7-29.

The modified bacterial strains which contain one of the
nucleotide sequences defined above or also a recombinant expression
vector and cloning previously defined, and preferably the plasmid
pHT671 or the plasmid pHT71, also enter into the framework of the
invention.

The invention also relates to a polypeptide toxic towards
larvae of Lepidoptera and in a preferential manner towards
S.littoralis, which attack cotton leaves or other crops such as those
listed above, characterized in that it is capable of forming an
immunological complex with antibodies directed against polypeptides
with larvicidal activity towards S.littoralis.

The invention relates more particularly to the NH_2-terminal
part of this polypeptide which contains the larvicidal activity.

The extremity of the active NH_2-terminal part corresponds
to the sequence (II) of amino acids given above.

A preferred polypeptide of the invention is that which
corresponds to this sequence (II) and corresponds to the sequence
(IV) of amino acids given in the preceding pages. This polypeptide
corresponding to the sequence (IV) contains 823 amino acids. Its
calculated molecular mass is 92906 Da.

16

This sequence of δ-endotoxin was compared with amino acid
sequences of δ-endotoxins derived from other strains of B.thuringiensis
active on the Lepidoptera and the genes of which have been isolated
and sequenced previously : the δ-endotoxins in question are those
of the strains kurstaki HD1 (19), kurstaki HD73 (20), berliner 1715
(21) and (22) Sotto (23) and aizawai IPL7 (24).

The results of these comparisons indicate that all are
different in the second quarter of the molecule (amino acids 281 to
620) whereas the NH_2-terminal part (amino acids 1 to 280) and the
COOH-terminal domain (amino acids 621 to 1175) of the protein are
highly conserved and differ only by several amino acids. On the other
hand, the δ-endotoxin corresponding to the sequence (IV) above shows
considerable differences from the other δ-endotoxins both in the
NH_2-terminal part (amino acids 1 to 280) and in the second quarter
of the molecule (amino acids 281 to 620). The results of these
comparisons indicate again that the NH_2-terminal half of the molecule
(amino acids 1 to 620) which corresponds to the toxic fraction of
the protein only show 46% homology with the other δ-endotoxins. The
most important differences are located in the second half of the toxic
part of the molecule (amino acids 281 to 620) with only 36% of
identical amino acids, the NH_2-terminal part (amino acids 1 to 280)
itself showing 58% of amino acids identical with the other δ-endo-
toxins. Such considerable differences have never been observed up
to now in the NH_2-terminal part of the toxic fraction of the molecule
among the δ-endotoxins active on the Lepidoptera.

In order to obtain a sequence of nucleotides entering into
the framework of the invention, coding for at least the active part
of a polypeptide showing a specific toxicity towards larvae of
Lepidoptera of the Noctuidae family, and preferably towards
S.littoralis, recourse is had, in conformity with the invention, to
the following steps, namely:
- the carrying out of a molecular hybridization between, on the one
hand, a nucleotide sequence of a strain of B.thuringiensis active
against S.littoralis and, on the other, at least two nucleotide
sequences, used as probes, derived from the 5' part of a restriction

17

fragment of a gene for δ-endotoxin of B.thuringiensis, this part coding
for the NH$_2$-terminal part of the polypeptide active on the larvae of
Lepidoptera, and from the 3' part of this fragment coding for the
COOH part of the polypeptide,

5   - the isolation of the hybrid fragment,
       - its cloning in a vector, followed by its purification.

       In an advantageous manner, the hybridization probes utilized
are obtained from a gene for the ζ-endotoxin derived from the strain
aizawai 7-29 coding for a protein of 130 kDa, active against
10   P.brassicae and inactive towards S.littoralis, this gene having been
cloned in the recombinant plasmid pHTA2.

       In an embodiment of the preceding procedure, the fragment
recombined with the vector in the cloning step is elaborated from
a HindIII-PstI restriction fragment derived from a single strain of
15   B.thuringiensis, preferably aizawai 7-29. In particular, this fragment
is carried preferentially by the recombinant plasmid pHTA6 such as
isolated with the aid of a probe constituted by a PvuII fragment of
2 kb of the plasmid pBT15-88 corresponding to the internal part of
a gene for the chromosomal crystal of the strain berliner 1715,
20   starting from transforming clones containing nucleotide sequences
derived from B.thuringiensis strains active against larvae of
Lepidoptera, inter-alia of S.littoralis.

       In another embodiment of the invention, the fragment
recombined with the vector in the cloning step is elaborated from
25   several sequences of nucleotides derived from recombinant vectors
containing sequences of nucleotides from at least two different strains
of B.thuringiensis, possessing the same restriction maps and themselves
containing all or part of the sequences of nucleotides capable of
coding for a polypeptide active, in a preferential manner, against
30   S.littoralis.

       In this case, the recombined fragment used in the cloning
step is a fragment of about 3 kb, advantageously elaborated from a
HindIII-HincII restriction fragment of about 1.1 kb derived from the
entomocidus 6-01 strain and a HincII-PstI fragment of about 1.9 kb
35   from the aizawai 7-29 strain. It corresponds to a truncated gene for
δ-endotoxin.

18

The HindIII-HincII and HincII-PstI restriction fragments
are carried more especially by the respective recombinant plasmids
pHTE6 and pHTA6 such as isolated with the aid of the probe constituted
by the PvuII fragment mentioned above.

5   The study of the toxicity towards the larvae of Lepidoptera
of the bacterial strains modified with the aid of the sequences of
nucleotides defined above, has made it possible to demonstrate their
high toxic activity, in particular with regard to the caterpillars
of S.littoralis.

10   This activity was estimated from the point of view of the
specificity index corresponding to the ratio

$$\frac{\text{LC50 S.littoralis}}{\text{LC50 P.brassicae}}$$

15 in which "LC50" represents the lethal concentration killing 50% of
the larvae in 72 hours.

The utilization of such an index makes it possible to
evaluate the activity of the bacterial strains studied without having
to consider the level of expression of the polypeptides.

20   The results obtained, which are reported in the examples
which follow, and the values of LD50 which are deduced from them,
have shown that the bacterial strains modified according to the
invention show a more specific toxic activity towards S.littoralis
than the native crystal proteins of the strains aizawai 7-29 or

25 berliner 1715.

Therefore, the invention relates to the use of these modified
strains, of recombinant vectors containing the nucleotide sequences
defined above, in particular the plasmid pHT671 and the plasmid pHT71,
and these sequences themselves for the elaboration of larvicidal

30 compositions preferentially toxic towards S.littoralis.

The larvicidal compositions of the invention are thus
characterized in that they contain an efficaceous quantity of
polypeptides such as defined above or expressed by the nucleotide
sequences mentioned above.

35   In order to produce these polypeptides the truncated genes

19

for δ-endotoxin corresponding to the nucleotide sequences of the invention are advantageously implemented.

These genes can be used to produce in excess the toxic polypeptide in microorganisms permitting the expression of the above recombinant vectors. Suitable strains of microorganisms include E.coli or also B.subtilis.

These truncated genes may be reintroduced into the strains of B.thuringiensis or into related species such as B.cereus, according to the standard techniques, for example, by transformation, conjugation or transduction. These techniques make it possible to produce the toxic polypeptide in large quantity without first having to modify the natural region of the promoter for the δ-endotoxin genes of B.thuringiensis.

This transformation may be carried out by using methods derived from the transformation of the protoplasts of B.subtilis according to (11) or of the vegetative cells of B.thuringiensis as described in (12).

The introduction of recombinant plasmids by a system of the conjugation type may be carried out by using B.thuringiensis as host strain and B.subtilis or Streptococcus faecalis as strains of the donor type by operating according to (13) and (14).

As a variant, the sequences of nucleotides are introduced into microorganisms living in the environment or in association with the plants and capable of expressing recombinant vectors containing these sequences. The introduction may be carried out in microorganisms such as Pseudomonas by working according to the procedure described in (17), by the intermediary of plasmid vectors containing the transposon Tn5 and the gene for the toxin, or Azospirillum or Rhizobium by means of the intermediary of suicide vectors derived from the plasmid RP4 and of mobilizing plasmids functional in Gram negative bacteria (for example, pRK2013) according to the procedures described in (18).

The truncated genes are alone in the strains of Bacilli or, as a variant, are associated with different δ-endotoxin genes which makes it possible to obtain crystals synthesized by these strains

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specifically toxic towards given species of Noctuidae, or toxic both
towards the Noctuidae and insects sensitive to other δ-endotoxins.
These recombinations, carried out _in vitro_ or _in vivo_ with the
nucleotide sequences of the invention and other δ-endotoxin genes

5   showing different toxic specificities, lead to the construction of
new genes coding for novel hybrid toxic proteins exhibiting a large
spectrum of activity towards insects. These new genes and these novel
proteins also enter into the framework of the invention.

         In these applications, the nucleotide sequences of the

10   invention may be transferred and expressed in plants sensitive to
S.littoralis in order to diminish the devastation caused by these
insects.

         Among the plants to be protected, mention should be made
of : cotton, clover, the tomatoe and alfalfa.

15   The transfer of the truncated gene into cotton plants may
be carried out by transformation involving strains such as
Agrobacterium as described in (15).

         In addition, the invention relates to the plant cells, the
plants and the seeds containing the nucleotide sequences defined above.

20   The plant cells according to the invention are cells, the
genome of which after transformation by a non-essentially biological
procedure possesses in a stable manner a sequence of nucleotides capable
of expressing a polypeptide toxic towards S.littoralis, such as that
defined above. The invention also relates to the plant cells derived

25   from their division.

         The plants according to the invention are plants transformed
by a non-essentially biological procedure, having in particular as
predator S.littoralis, the genome of which possesses in a stable manner
a sequence of nucleotides such as that defined above, capable of

30   expressing a polypeptide toxic towards S.littoralis. The plants in
question are also plants derived from their reproduction, their
multiplication or hybrid crosses.

         In accordance with another feature, the invention relates
to plants having in particular as predator S.littoralis, possessing

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21

in addition to their initial phenotypic and genotypic characters
the property of expressing a polypeptide toxic preferentially towards
S.littoralis, this property resulting from the insertion in their
genome by means of genetic manipulation of a sequence of nucleotides
capable of expressing the said polypeptide.

In addition, the invention relates to seeds capable of giving
rise to a plant such as that defined above or derived from such a
plant, characterized in that they have integrated into their genome
by genetic manipulation a nucleotide sequence described above.

Other characteristics and advantages of the invention will
become apparent in the course of the description and in referring
to the examples in which :

- figure 1 presents the restriction map of the plasmids pHTA6 and
pHTE6,

- figure 2, the restriction map of a gene for a crystal protein of
the aizawai 7-29 strain cloned in the plasmid pHTA2 and defining the
DNA fragments which are used as probe,

- figure 3 shows the fragment of 6.6 kb cloned in pHTA6 and the result
of a hybridization carried out between this fragment and the probes
described in figure 2,

- figure 4, the restriction map of the plasmid pHT671, and

- figure 5, the photographs of the immunodiffusion tests.

The hybridization experiments carried out for the
implementation of the invention were performed at 42°C for 24 h in
a solution containing 5 x SSC, 30% formamide and 1 Denhardt (7) in
the presence of the DNA probe labelled with ^{32}P. The filters are washed
at 42°C, 20 mn, by using successively the following solutions :
5 x SSC in 30% formamide, 5 x SSC, 2 x SSC, 1 x SSC and 0.5 x SSC
before drying at room temperature.

EXAMPLE 1 - Construction of a DNA sequence of about 3 kb containing
a hybrid gene of an insecticidal toxin.

This construction comprises :

1/ the preparation of gene banks of B.thuringiensis

2/ the selection and characterization of transforming clones containing
the genes of a crystal protein and nucleotide sequences responsible

22

for the larvicidal activity,

3/ in vitro recombination of these sequences in a cloning vector with construction of the plasmid pHT671.

These different steps are carried out as follows :

1/ Preparation of gene banks of B.thuringiensis.

The total DNA of the aizawai 7-29 and entomocidus 6-01 strains of Bacillus thuringiensis is purified by using the method reported in (1) and 50 µg of each purified DNA are completely digested with the restriction enzyme PstI.

The DNA digested by PstI is analysed by horizontal electro-phoresis on a 0.8% agarose gel and DNA fragments of a size of 5 to 8 kb are recovered from the agarose gels by electroelution in a manner described in (2).

The purified DNA fragments of 5-8 kb of the aizawai 7-29 strain are ligated to the DNA of the cloning vector pUC18 digested by PstI according to (3).

The purified DNA fragments of 5-8 kb of the entomocidus 6-01 chain are ligated to the DNA of the cloning vector pUC9 digested by PstI. The cells of E.coli JM83 are transformed with the ligation mixture as described in (4).

The transforming clones of E.coli are selected on LB medium containing 100 µg/ml of ampicillin.

2/ Isolation and characterization of the transforming clones containing the genes for a crystal protein.

A/   Screening of the transformed E.coli cells with the aid of an internal fragment of a gene of the crystal protein labelled with ^{32}P, used as probe :

Transforming clones containing recombinant plasmids carrying the gene for the crystal are detected by colony hybridization according to the method described in (5), by using as probe a PvuII fragment of 2 kb of the pBT 15-88 plasmid corresponding to an internal part of the gene for the crystal protein located on the chromosome of the berliner 1715 strain.

B/   Characterization of the recombinant plasmids present in the clones which react with the above probe.

Two recombinant plasmids, pHTA6 and pHTE6, isolated
respectively from gene banks constructed from the strains aizawai
7-29 and entomocidus 6-01, show a homology with this probe. In each
case, a DNA fragment of about 6.6 kb was cloned.

5   The restriction map of the two plasmids is given in figure
1. The comparison of the restriction sites shows that the two DNA
fragments cloned appear to be identical.

        In order to delimit the sequences corresponding to the gene
for the δ-endotoxin, different DNA fragments labelled with ^{32}P, derived
10   from a gene of the crystal previously characterized, and cloned in
the recombinant plasmid pHTA2, are utilized as probes. This latter
gene for the crystal also derived from the aizawai 7-29 strain codes
for a protein of 130 kd active against P.brassicae but not against
S.littoralis. This type of gene possesses the same restriction map
15   as the gene for the δ-endotoxin derived from the berliner 1715 strain.
In figure 2 is shown the restriction map of this gene for the crystal
protein of the aizawai 7-29 strain cloned in the plasmid pHTA2. The
thick lines shown above the map correspond to the fragments used as
hybridization probes.

20   The plasmids pHTA6 and pHTE6 are hydrolysed by different
restriction endonucleases, analysed by horizontal electrophoresis
on a 0.8% agarose gel and hybridized with the different probes.

        The transfer of the DNA to nitrocellulose filters is carried
out according to the method of SOUTHERN described in (6). The
25   hybridization is conducted at 42°C for 24 hours in a solution
containing : 5 x SSC, 30% formamide and a 1x Denhardt mixture described
in (7) in the presence of a DNA probe labelled with ^{32}P. The filters
are then washed at 42°C for 20 minutes, by using successively the
following solutions : 5 SSC in 50% formamide, 5 SSC, 2 SSC, 1 SSC
30   and 0.5 SSC before being dried at room temperature.

        The results of these hybridization experiments are summarized
in figure 3. It appears that each extremity of the cloned DNA fragments
of 6.6 kb shows a homology with the probes. The PstI-KpnI fragment
of 1.5 kb reacting with the probe No. 3 corresponds to the 3' end
35   of a gene of the crystal protein present in both the aizawai 7-29

and <u>entomocidus</u> 6-01 strains. As pointed out in figure 3, the probes
No. 1 and No. 2 corresponding to the 5' end of the gene for the
δ-endotoxin of pHTA2 hybridize with the <u>HindIII-HincII</u> fragment of
1.1 kb contained in the plasmid pHTA6. The probe No. 3 which covers
the 3' end of the gene of the δ-endotoxin of pHTA2 hybridizes with
the <u>HindIII-PstI</u> fragment of 0.4 kb contained in the plasmid pHTA6.
It should be noted that a weak hybridization signal is obtained with
the probe No. 2 whereas the two other probes give easily detectable
signals.

From these results, the inventors have established that
the <u>HindIII-PstI</u> DNA fragment of 3 kb corresponds to a large part
of a gene for the δ-endotoxin which commences close to the central
<u>HindIII</u> site. It seems clear in the light of results of the
hybridization experiments that the gene for the δ-endotoxin shows
substantial differences from those characterized in the prior art.
On the basis of these results it was decided to clone the <u>HindIII-
PstI</u> fragment of 3 kb in the vector pUC9.

3/ <u>Construction of the plasmid pHT 671 containing a hybrid gene of
the reconstituted insecticidal toxin.</u>

The <u>HindIII-HincII</u> DNA fragment of 1.1 kb derived from the
plasmid pHTE6 and the <u>HincII-PstI</u> DNA fragment of 1.9 kb derived from
the plasmid pHTA6 are purified on agarose gels.

Equal amounts of the two purified DNA fragments and the
DNA of pUC9 digested with <u>HindIII</u> and <u>PstI</u> are mixed and ligated.
The ligation mixture is used to transform competent cells of <u>E.coli</u>
JM83, then the transformed <u>E.coli</u> cells are selected on LB medium
containing ampicillin. One of the interesting recombinant clones
examined contains a plasmid designated by pHT671, the restriction
map of which was determined and is shown in figure 4. This plasmid
(pHT671) contains a DNA fragment of 3 kb inserted in the vector pUC9.
This DNA sequence has the same restriction map as the <u>HindIII-PstI</u>
fragments of 3 kb contained in the plasmids pHTA6 and pHTE6, but
corresponds to a reconstituted DNA molecule constructed by <u>in vitro</u>
recombination from DNA sequences derived from the <u>aizawai</u> 7-29 strains
on the one hand and <u>entomocidus</u> 6-01 on the other.

EXAMPLE II : <u>Study of the nucleotide sequence of the promoter region</u>
<u>and of the region coding for the NH$_2$-terminal part of the δ-endotoxin</u>
<u>active against the Noctuidae</u>.

The <u>HindIII</u>-<u>HincII</u> fragment of pHT671 is sequenced in

5   conformity with the method described in (8) by using a M13 system.
In order to obtain partially overlapping cloned DNA fragments which
will be used in the sequencing of the DNA, recourse is had to the
method of subcloning by deletion in M13, developed by DALE et al (9).

The sequence of 940 nucleotides of the <u>HindIII</u>-<u>HincII</u>

10   fragment which has a length of about 1 kilobase corresponds to the
chain arrangement I above.

The analysis of this sequence shows that the largest open
reading frame starts at position 241 and that a potential site of
binding to the ribosomes, GGAGG, is found six base pairs upstream

15   from this ATG codon (position 230 to 235). The region localized between
the nucleotides 137 and 177 (position -103 to -63 upstream from the
ATG codon) is strongly homologous with the region present upstream
from the gene for the crystal of the strain <u>kurstaki</u> HD1 Dipel (BTK)
sequenced by WONG et al (1983) and described in (16) and the authors

20   of which have shown that it contains three promoters <u>BtI</u>, <u>BtII</u>, and
<u>Ec</u>, functional in <u>B.thuringiensis</u> and <u>E.coli</u>, respectively. The
comparison between the amino acid sequences deduced from the first
750 nucleotides of the genes of BTK and pHT671, show that these
polypeptides exhibit significant differences at the level of the

25   N-terminal half of the active part derived from the protoxin (only
66% strict homology). It is important to note that it is the first
time that a gene for the δ-endotoxin isolated from a strain active
against the Lepidoptera codes for a polypeptide which shows substantial
differences in this region. In fact, this N-terminal domain appears

30   to be strongly conserved (more than 97% of strict homology) among
all of the genes for the crystal active on Lepidoptera which have
been sequenced hitherto. Moreover, the inventors have shown that the
degree of variability is of the same order if the nucleotide sequences
of pHT671 and other genes of the Lepidoptera type are considered.

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EXAMPLE III : Construction of a DNA sequence of about 2.7 kb containing
a gene for a larvicidal toxin.

In order to achieve this construction the DNA of the aizawai
7-29 strain of B.thuringiensis was used up to the step for the
production of the plasmid pHTA6 as described in Example I.

The HindIII-PstI fragment of about 2.7 kb obtained from
the plasmid pHTA6 was then subcloned in the vector pUC9, previously
hydrolysed by the restriction enzymes HindIII-PstI in order to give
the plasmid pHT71.

EXAMPLE IV : Study of the sequence of nucleotides constituting the
plasmid pHT71 coding for a polypeptide toxic towards the larvae of
Lepidoptera of the family of the Noctuidae.

The HindIII-PstI fragment of 2.7 kb of pHTA6, which was
subcloned in pHT71, was sequenced by means of the technique of Sanger
et al. (8) using the phage M13mp19 and the subcloning system by
deletions developed by Dale et al (9). This system makes it possible
to obtain M13 phages containing a series of partially overlapping
DNA fragments which can be utilized for sequencing the DNA.

The sequence of nucleotides of this 2.7 kb fragment which
corresponds to the chain arrangement (III) given above, was determined
on the 2 DNA strands, with the exception of the last 212 nucleotides
(position 2500 to 2711) which were sequenced only on a single strand.

The nucleotide sequence of this HindIII-PstI fragment has
a length of 2711 nucleotides. This fragment contains the potential
promoter as well as the largest part of the gene for the δ-endotoxin
active on S.littoralis.

EXAMPLE V : Study of the specific toxicity of the recombinant clones
of E. coli JM83 (pHT671) and JM83 (pHT71) against S.littoralis.

The toxicity of the recombinant clones of E.coli JM83
containing pHT671 and of E.coli JM83 containing pHT71 was determined
by biological tests on caterpillars of the P.brassicae and S.littoralis
species as described by LECADET and MARTOURET in (10). The results
were compared with the specific toxicity of the native crystal proteins
purified from the strains berliner 1715 and aizawai 7-29, entomocidus
6-01 B.cereus 569 (containing the plasmid pBT45, pAMβ1) against the

two species of insects. The specific toxicity of the recombinant clone
and of the strains of B.thuringiensis is expressed in terms of
"specificity index" previously defined.

The results obtained are reported in table 1 below.

5   In this table, for E.coli strains, the concentration 1
corresponds to a 14 hours bacterial culture concentrated 20 times,
disintegrated by ultrasound ; for the B.thuringiensis strains the
concentration is expressed in µg of crystal protein per µl of
preparation. The toxic activity of the preparations was tested by
10   the forced ingestion with 5 µl of preparation on caterpillars at the
fifth stage of development, or by a technique of free ingestion
utilizing larvae at the second stage of development.

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TABLE 1

Comparative toxicity of a recombinant clone and two strains
of B. thuringiensis towards S. littoralis and P. brassicae.

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Strains and plasmids	S.littoralis		P.brassicae	Specificity index $\dfrac{\text{LC50 S.littoralis}}{\text{LC50 P.brassica}}$
	LC50 2nd larval stage	LC50 5th larval stage	LC50 5th larval stage	
JM83 (pUC18)	> 1	> 1	> 1	-
JM83 (pHT671)	0,04	0,13	0,72	0,2
JM83 (pHTA2)	> 1	> 1	0,03	> 30
JM83 (pHTA4)	> 1	> 1	> 1	-
JM83 (pHT71)	ND	0.5	> 1	< 0.5
berliner 1715 native crystals	ND	0.11	0.007	15.7
aizawai 7.29 native crystals	ND	0.02	0.04	0.5
entomocidus 601 native crystals	ND	0.028	0.012	2.3
B.cereus 569 (pBT45,pAMβ1)	ND	0,38	0,054	7

Examination of the LC50 values summarized in this table 1 shows that the protein extracts of the recombinant clones JM83 (pHT671) and JM83 (pHT71) are preferentially toxic against S.littoralis. Secondly, a comparison of the values of the specificity index shows that the larvicidal activity of the recombinant clones is more specific by a factor of 2.5 times towards S.littoralis than the native crystal proteins of the aizawai strain. Moreover, the recombinant clones of JM83 (pHT671) and JM83 (pHT71) are very active against another insect of the family of the Noctuidae, Mamestra brassicae (in the case of the clone JM83 (pHT671) for example, the LC50 value is 0.02, utilizing larvae at the second stage of development).

These two results show that the gene for the larvicidal toxin constructed and cloned in the plasmids pHT671 and pHT71 codes for a protein specifically active against S.littoralis.

Other preparations obtained from recombinant clones containing plasmids carrying genes coding for other types of δ-endotoxins (pHTA2 and pHTA4) are not active on S.littoralis : it may be seen that the plasmid pHTA2 codes for a δ-endotoxin specifically active on P.brassicae whereas the plasmid pHTA4 codes for a δ-endo-toxin, the insect target for which has not yet been identified. It can also be seen that the crystalline inclusions produced by a strain of Bacillus cereus which has received the plasmid pBT45, one of the plasmids of the aizawai 7-29 strain which also carries a δ-endotoxin gene (the gene of plasmid origin of the aizawai 7-29 strain), are also specifically active on P.brassicae.

Similar results are obtained by using, in the place of crude bacterial extracts, soluble protein extracts prepared from different recombinant clones of E.coli.

On the basis of the LC50 values reported in the table above and a mean individual weight of 41 mg per L5 larva (fifth larval stage) of S.littoralis, the value of the LD50 was estimated at 2.4 µg/gram of larva for the native crystals of the aizawai 7-29 strain.

On these same bases and on the basis of equivalence factors making it possible to pass from the total bacterial mass to the

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quantity of specific proteins (about 2% of the total proteins in E.coli JM83 (pHT671), the LD50 corresponding to the toxin produced by the expression in E.coli JM83 of the gene according to the invention cloned in the plasmid pHT671, was determined and estimated at a value close to 5.5 to 6 µg/gram of larva.

On these same bases and after determination of the LC50 of soluble protein extracts prepared from ground cultures of E.coli JM83 (pHT671), the value of the LD50 corresponding to the toxin present in these extracts was estimated at 4.15 µg/gram of larva.

In the two cases and particularly in the case of the ground preparations of E.coli, the calculated values of LD50 are approximate and higher than that of the native crystals, because it is not a question of a purified toxin. However, these data indicate without ambiguity that the gene expressed by pHT671 specifies a δ-endotoxin exhibiting the specificity towards S.littoralis. In fact, the same type of estimation made with extract of E.coli JM83 (pHTA2) carrying a δ-endotoxin gene of different specificity leads to values 30 to 50 times higher than the LD50 of the soluble extracts towards S.littoralis (135 to 350 µg/gram of larva).

The foregoing data will easily make it possible for the person skilled in the art to develop active larvicidal compositions with the proteins of the invention.

Other toxicity experiments were carried out utilizing larvae of M.brassicae, S.fruqiperda and S.littoralis at the second larval stage. The results obtained, expressed in terms of LC50 as defined for table 1, are given in table 2.

TABLE 2

ACTIVITY OF THE RECOMBINANT CLONES AGAINST THE LARVAE OF INSECTS OF THE FAMILY OF THE NOCTUIDAE: M. BRASSICAE, S. FRUGIPERDA, and S. LITTORALIS.

STRAINS AND PLASMIDS	INSECT LARVAE AND STAGE	M. BRASSICAE	S. FRUGIPERDA	S. LITTORALIS
		LC50 2nd STAGE	LC50 2nd STAGE	LC50 2nd STAGE
JM 83 (pUC18)		NT	NT	NT
JM 83 (pHTA2)		> 1	0,51	0,9
JM 83 (pHT671)		0,02	0,5	0,03
JM 83 (pHT71)		ND	ND	0,0
JM 83 (pHTA4)		> 1	0,54	> 1

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It emerges from the examination of table 2 that the crude
bacterial extracts of the recombinant clone JM83 (pHT671) are toxic
towards M.brassicae and S.littoralis (the values of LC50 are 0.02
and 0.03, respectively) and weakly toxic towards S.fruqiperda (LC50
5   of 0.5).

The extracts of the recombinant clone E.coli JM83 (pHTA2)
are weakly active towards S.fruqiperda and S.littoralis and not at
all toxic towards M.brassicae. The extracts of the recombinant clone
JM83 (pHTA4) are not toxic towards M.brassicae and S.littoralis and
10   are weakly toxic toward S.fruqiperda.

These results confirm the high specific toxicity of the
proteins obtained from pHT71 and pHT671 towards S.littoralis and
show that this class of crystal protein is also very active towards
M.brassicae.

15   EXAMPLE VI : Study of the specificity of the polypeptides expressed
by the clones formed by introduction of the plasmids pHT671 and pHT71
into E.coli.

This study was carried out owing to immuno-diffusion tests.
The results are reported in figure 5 (which includes figures 5A and
20   5B).

The implementation of the immuno-diffusion experiment was
done in conformity with the following protocol :

Soluble extracts of proteins of E.coli clones containing
the plasmids pHT671, pHTA4, pHTA2 or pHT71, pUC18 were placed in
25   the wells Nos. 2, 3, 4, 5, 6, respectively. A sample of a solubilized
purified crystal of aizawai 7-29 was placed in the well No. 1 in
order to serve as positive control.

In the test recorded in figure 5A an antiserum against
all of the δ-endotoxins of aizawai 7-29, containing rabbit antibodies
30   directed against the solubilized crystal proteins, was used and placed
in the central well.

An immunoprecipitation line was observed in all of the
cases except in the case of the extract of E.coli containing only
the plasmid vector (well No. 6).

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It was observed that the immuno-precipitation lines derived
from the wells No. 4 and No. 5 cross, which shows that the products
encoded by the plasmids pHTA2 and pHT71, respectively, display
different antigenic determinants.

5   In the test recorded in figure 5B, the anti-serum used
contained rabbit polyclonal antibodies against the crystal proteins
of berliner 1715.

           An immunoprecipitation line was observed with the extracts
of E.coli JM83 (pHTA4) (well No. 3) JM83 (pHTA2) (well No. 4). On
10   the other hand, the E.coli clones JM83 (pHT71) (well No. 5), JM83
(pHT671) (well No. 2) or JM83 (pUC9) (well No. 6) did not give immuno-
precipitation.

           It may be deduced from that that the genes for the crystal
isolated in pHTA4 and pHTA2 express polypeptides having antigenic
15   determinants in common with the proteins of the crystal of berliner
1715, a strain which is not specifically active towards S.littoralis.

           On the other hand, the crude extracts of E.coli containing
the plasmids pHT671 and pHT71 contain polypeptides having antigenic
determinants in common with the crystal proteins of the aizawai 7-29
20   strain, which are not related immunogenically with the crystal proteins
of the berliner 1715 strain.

           These results confirm those given previously with respect
to the specificity of the genes isolated in the plasmids pHT71 and
pHT671.

25   Antigen-antibody precipitation assays have made it possible
to determine the level of expression of the δ-endotoxin genes in
different recombinant clones.

           The results obtained have shown that the crystal protein
represents between 7 and 10% of the total cellular proteins of E.coli
30   JM83 (pHTA2), between 2 and 3% in E.coli JM83 (pHT671) and between
0.5 and 1% in E.coli JM83 (pHTA4) and E.coli JM83 (pHT71).

35

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